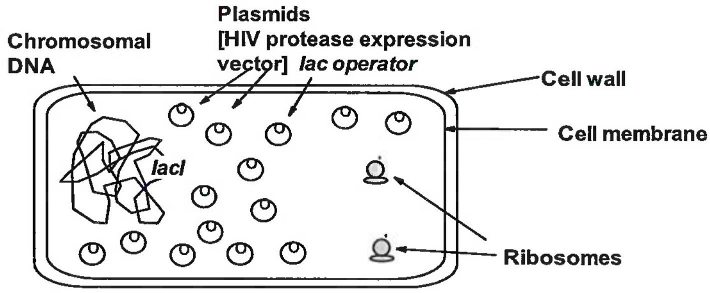
Lecture 39: Lac and T7 Expression Vectors

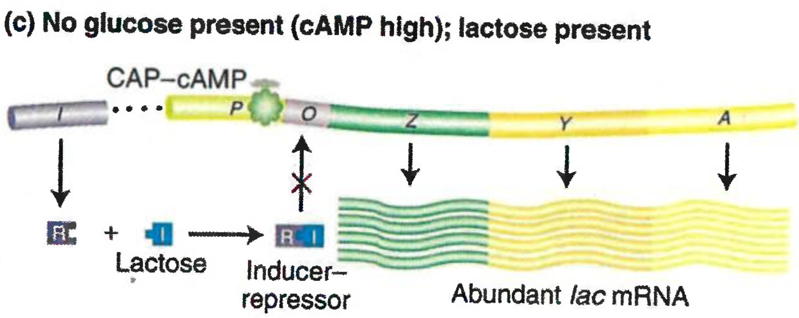
Lac Expression Vectors - Inducible

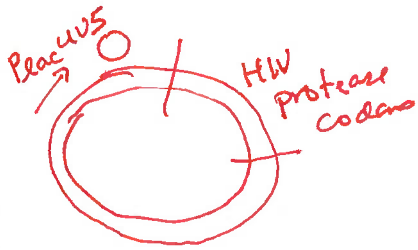
Expression of Recombinant Proteins:

- **Lac operator** – DNA sequence that binds the lac repressor protein, utilized as an on/off switch in the control of recombinant proteins.
- **Lac repressor** – protein that binds to the lac operator, released from DNA by IPTG
- **Lac I gene** – chromosomal, produces the lac repressor protein
- **LacUV5 promoter** – Mutation of the lac promoter that increases RNAP activity, avoiding the need for CAP+cAMP binding to get high levels of transcription.



1. The constitutive expression of high levels of almost any protein is toxic to the bacteria. The protein itself may be toxic, or the simple competition for cellular resources can lead to poor growth of the bacterial host, and in some cases, cell death. Therefore, it is necessary to regulate the production of high levels of recombinant protein.
2. In the Lactose operon production of proteins involved in the metabolism of lactose are controlled by the binding of the **lac repressor** (a protein that is the product of the *lacI* gene, produced from the bacterial chromosome) to a region of the DNA near the promoter region of the genes that encode the proteins for lactose metabolism. This segment of DNA is called the **lac operator**. High-level expression from the lac operon only occurs when glucose is low, leading to high cAMP levels in the bacterial cytoplasm. cAMP binds to the catabolite activator protein (CAP), the CAP-cAMP complex binds upstream from the lac promotor enhancing transcription.





3. The lac operator system can be used it to control expression of any other gene by simply placing the appropriate DNA segments in the correct location in our expression vector. To avoid regulation by CAP, the normal lac promotor is replaced by the lacUV5 promoter, this gives high levels of expression even if glucose levels are high.

```
AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTAGGAAACAGCTATG..
   CAP binding                              Lac promotor
                                                                                                 Protein->
                                                                          mRNA-----------------------------------> 
AATGTAAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTAGGAAACAGCTATG..
                                    -35                 -10        <--lac operator--------->          Met...
                                         LacUV5 promoter
```

***Reflection**: why is it preferable to use the lacUV5 promotor instead of the normal lac promoter?*

use high glucose → optimal growth.

4. The lac repressor binds to the DNA when lactose is absent and blocks transcription of the DNA. When lactose is present, it binds to the lac repressor, causing an **allosteric** change that releases it from the DNA. Since lactose would be rapidly degraded by the bacteria, a non-hydrolyzable analog, isopropyl-thio-galactoside (IPTG), is used instead. Once the lac repressor leaves the DNA, RNA polymerase can bind, allowing production of mRNA that can be used by the ribosome to produce HIV protease.

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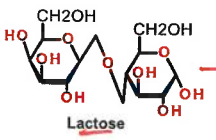
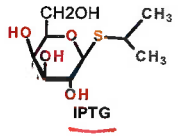
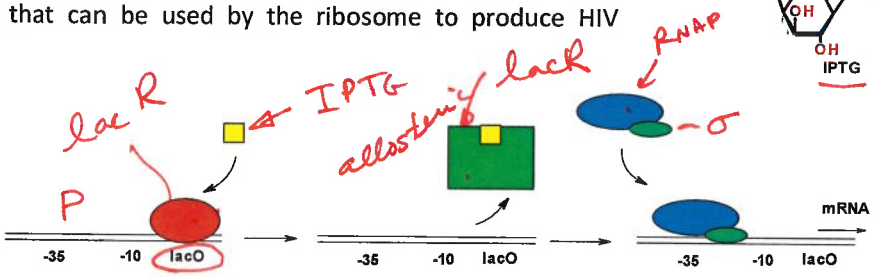
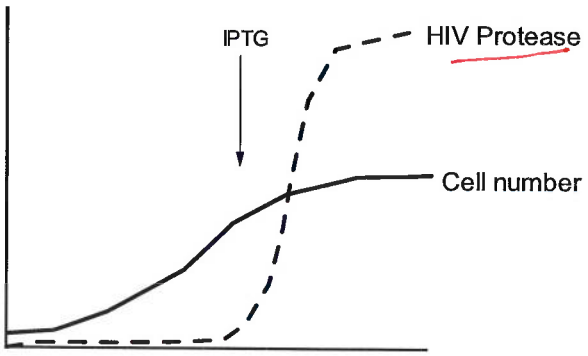


→ D metabolized



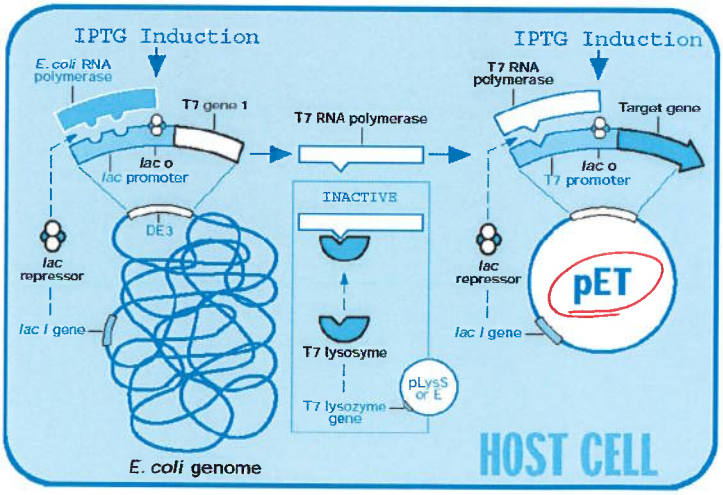
IPTG





Alternative Expression Systems: T7-pET vector Series.

- Use T7 phage promotor/polymerase. Only significant difference is a different promotor is used to drive production of mRNA and a specialized host strain is required that expresses T7 polymerase.
- T7 polymerase only recognizes T7 promoters, and is more active than E. coli RNA polymerase, producing more mRNA transcripts (and therefore more protein).
- T7 polymerase under lac control on the host E. coli chromosome. T7 polymerase only produced when IPTG is added. 1st level of control in expression.
- Target gene is usually also under lac control on the plasmid. 2nd level of control of expression.

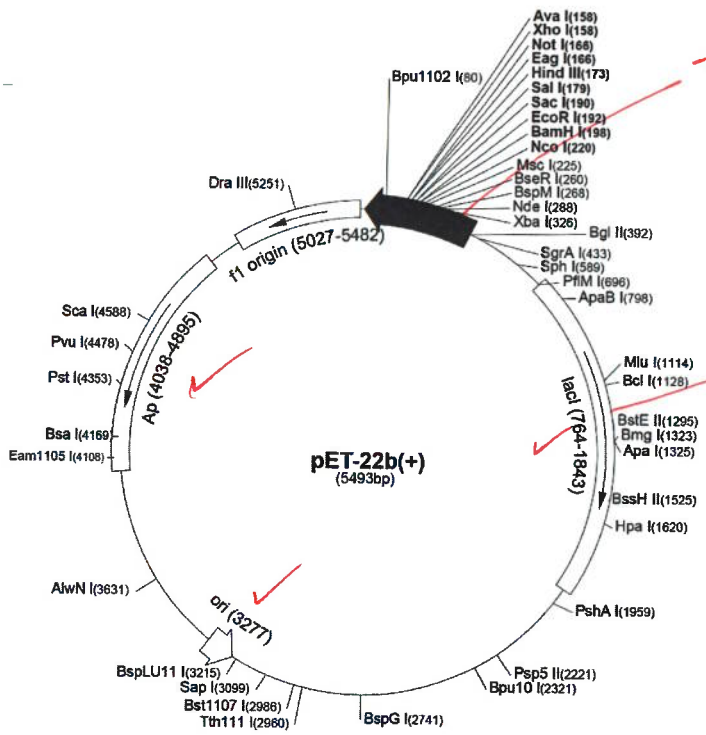


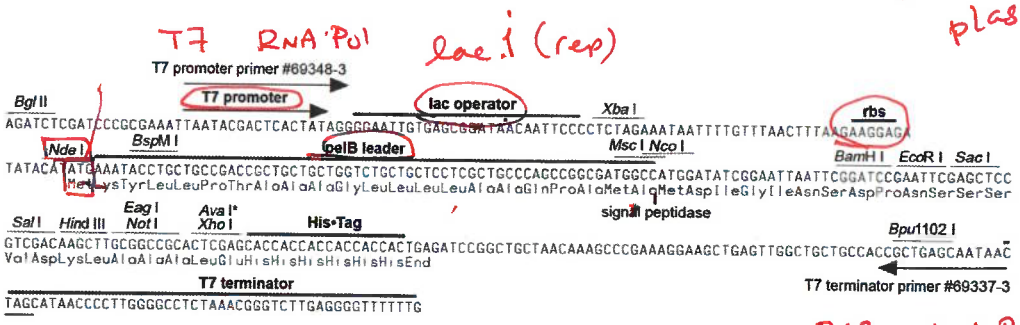
- Production can be attenuated by T7 lysozyme gene which inactivates T7 RNA polymerase.

i) IPTG

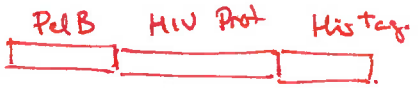
ii) produce T7 RNA Pol

iii) produce mRNA target gene (HIV protease)





pET-22b(+) cloning/expression region



1. Expression from the T7 promotor is under double lac control, the lac repressor blocks expression of T7 polymerase (on the chromosome) and expression of the gene on the plasmid.
2. Inserted codons using the NcoI site will add a signal peptide to the protein to cause expression from the cell.
3. A His-tag can also be added to the C-terminus of the protein, using the stop codon after the six His residues.

Cloning into pET22b using NcoI/XhoI would use both of these features. The PCR product that would be inserted would have the following sequence.

```
YYCCATGGXX[codon 1][codon 2].....CTCGAGZZ
YYGGTACC                         GAGCTCZZ
  NcoI                            Xho I
```

- The extra YY and ZZ bases allow NcoI and Xho I to efficiently cleave the DNA (many restriction enzymes cannot cleave if their site is at the very end of a fragment).
- The three bases (GXX) after the underlined **ATG** represent the 2nd codon, which is restricted to Val, Ala, Asp, Glu, Gly in order to have the NcoI site.

The final construct will be: -T7-lacO-rbs-Met—pelB---NcoI-coding region-Xho-HisHisHisHisHisHisTAA-----

PelB - Export of Recombinant Proteins.

A specialized *protein* sequence, called the leader peptide, when present as the amino terminal residues of the protein, signals the export of the protein out of the cell. This may reduce the toxicity of the protein and make purification easier since only a small number of proteins are exported out of the cell.

During the export process, this peptide is cleaved by the **leader peptidase** (also known as signal peptidase), producing the mature exported peptide.

The main features of this peptide are:

i) Basic residue at amino terminus

ii) Non-polar segment of ~15 amino acids.

iii) Cleavage site, which is followed by a second basic residue (---Ala^Arg---)

To use pET22b to express HIV protease we would modify our original PCR primers to add the NcoI and XhoI sites (instead of the EcoR1 and BamH1), after ligating into pET the final sequence, from the ribosome binding site to the stop codon is:

```
RBS           NdeI                                                                          NcoI                            XhoI
AAGGAGATATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGAT--HIV Protease---CTCGAGCACCACCACCACCACCACTGA
                 MetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaMetAspProGln-----AsnPheLeuGluHisHisHisHisHisHis
```

2) The peptide, as it comes off of the ribosome will look like:

```
-----------------Leader Sequence------------------------------------|-----HIV protease----|      |......His Tag....
fMetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaMetAspProGln-----AsnPheLeuGluHisHisHisHisHisHis
                                                                   ^Cleavage site for leader peptidase
```

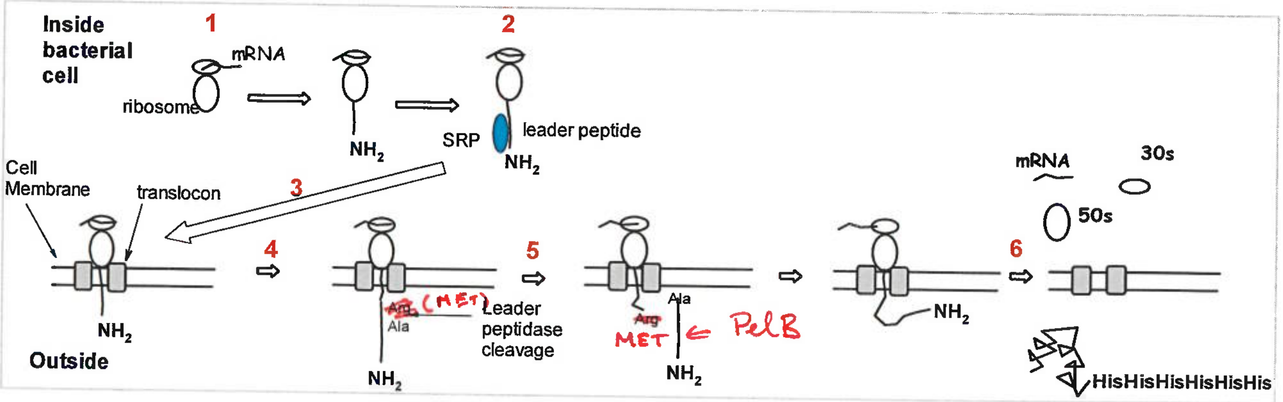
3) After export out of the cell the final product will be as follows (The bold amino acids were added as part of the NcoI and XhoI sites that were required to insert the PCR product into the expression vector.):

|---------**HIV protease**-------------| |---------**His Tag**-----------|

MetAspProGlnIle----LeuAsnPhe**LeuGlu**<u>HisHisHisHisHisHis</u>

Protein Export Machinery:

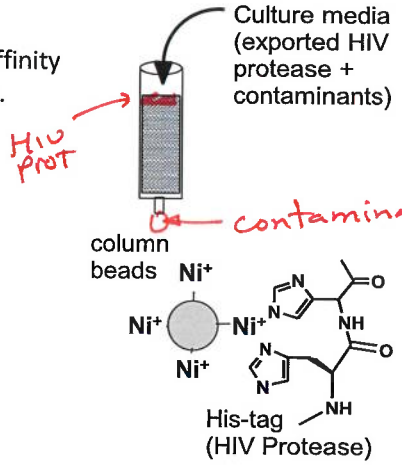
1. mRNA binds to the ribosome.
2. When leader peptide emerges from the ribosome it binds to the signal recognition particle (SRP).
3. The ribosome/mRNA/SRP binds to transport machinery(translocon) in the cell membrane.
4. Protein synthesis continues, protein extruded outside the cell.
5. Leader peptidase cleaves off leader peptide.
6. Protein is completely exported out of the cell.

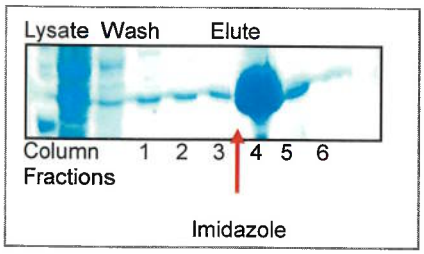


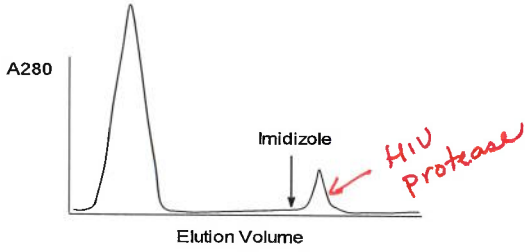
Note: The cleavage site for the peptidase is either Ala-Met, or Ala-Arg, Ala-Met is found in the PelB leader.

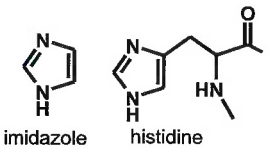
His Tag - Affinity Purification: One-step purification of HIV protease from culture media via affinity chromatography.

1. Culture media, containing HIV protease, is washed over metal ion affinity column (usually has Ni^{2+} or Co^{2+} attached to chromatography beads.
2. His_6-tag binds to immobilized nickel ions on chromatograph beads.
3. Impurities washed away.
4. Elution with imidazole (competes with His sidechain for Ni^{+}).

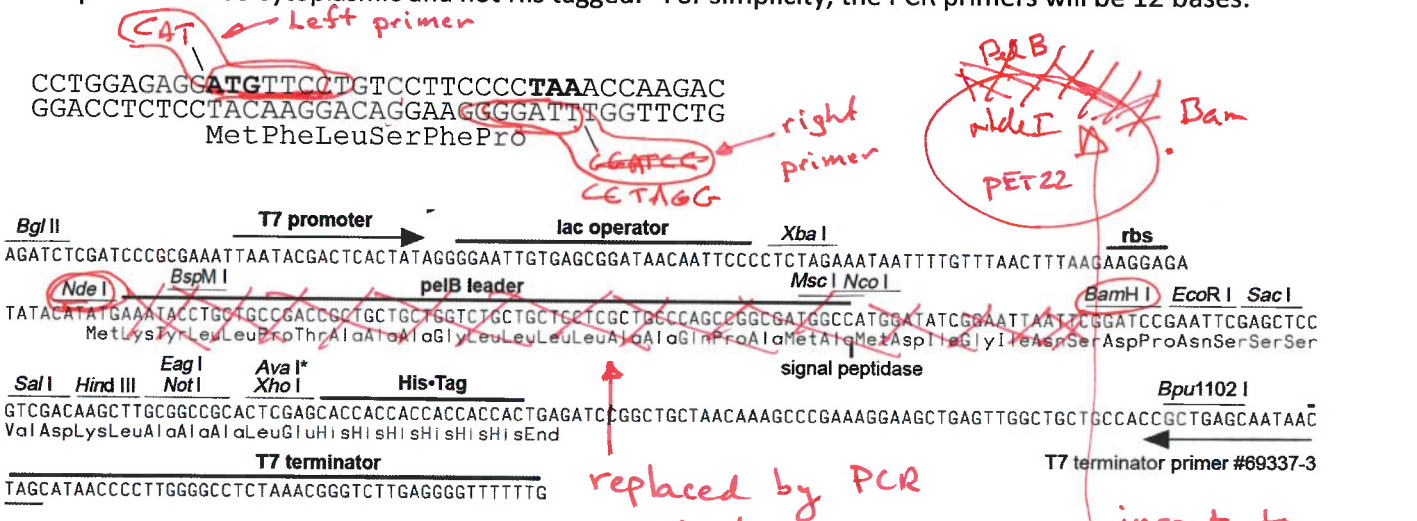






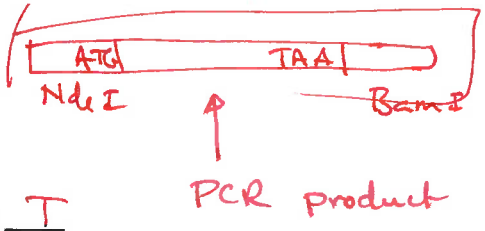


Example 1 – Given the following gene, what PCR primers would you use to clone into pET22 such that the protein will be cytoplasmic and not His tagged. For simplicity, the PCR primers will be 12 bases.



Approach:

1. Identify correct cloning sites.
2. Incorporate sites into PCR primers.
3. Check to make sure reading frame is correct



Left primer: C A T A T G T T C C T

Right primer: G G A T C C T T A G G

Start

BamHE STOP

Any site downstream from the Nde can be used to clone the PCR product (BspM1 through Bpu1102I) - I selected BamH1.

The stop codon that is part of the PCR product will terminate protein synthesis, so the His codons will never by read by the ribosome, there is no need to delete them.

Example 2 – Express human growth hormone in bacteria using pET-22b(+) such that it is exported out of the cell and has a His tag for purification. The sequence of human growth hormone is

Met-Val-Phe-Arg

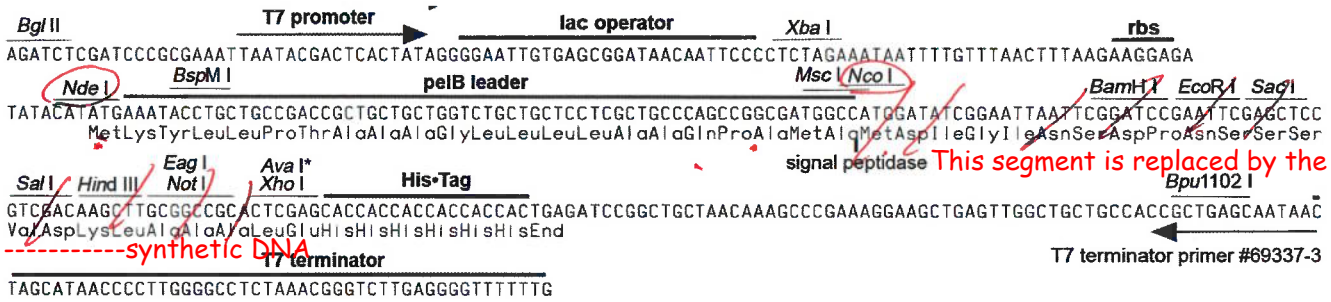
You should assume that you can have any DNA molecule synthesized.

1. What sites will you use on pET-22b? These sites have to be on the end of your synthetic DNA.
2. Convert the amino acid sequence to DNA to give your final DNA fragment that will be inserted into pET22 using restriction enzymes and ligase.

```
 NcoI            XhoI
 CCATGGTTTTTCGCCTCGAG
..AlaMetValPheArgLeuGluHis..
```

5' Base	Middle Base				3'
	T	C	A	G	
T	Phe	Ser	Tyr	Cys	T
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	**Term**	**Term**	A
	Leu	Ser	**Term**	Trp	G
C	Leu	Pro	His	Arg	T
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	T
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	T
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Final exported product, after cleavage by signal peptidase is: MetValPheArgLeuGluHisHisHisHisHisHis

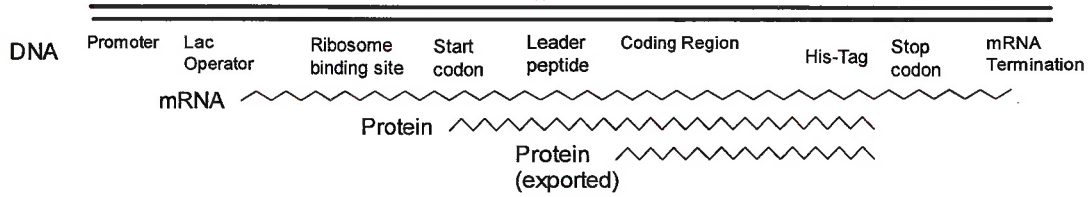


Post-Purification Processing: Note that the c-terminal sequence is modified, LeuGluHisHisHisHisHisHis has been added – this may affect function. The extra amino acids can be removed by incorporating a site for a protease. In this case, the Arg at the end is a substrate for trypsin, so the extra amino acids could be removed by treating the purified protein with trypsin, and the Histag peptide removed.

MetValPheArgLeuGluHisHisHisHisHisHis→ MetValPheArg LeuGluHisHisHisHisHisHis

Xho His6

Summary of Expression Features: Trypsin treatment releases the intact hormone



mRNA production (DNA to mRNA)	X	X							X
Protein production (mRNA to protein)			X	X	codons			X	
Protein export					X				
Protein purification							X		